Foxc2 overexpression enhances benefit of endothelial progenitor cells for inhibiting neointimal formation by promoting CXCR4-dependent homing

Dujuan Li, PhD, Dan Yan, PhD, Weishuo Liu, PhD, Mincai Li, PhD, Jun Yu, PhD, Yan Li, PhD, Zhiling Qu, MM, and **Qiurong Ruan, PhD,** *Wuhan, China*

Objective: **Endothelial progenitor cells (EPCs) are capable of enhancing re-endothelialization and attenuating neointimal formation. However, inefficient homing limits the therapeutic efficacy of EPCs transplantation. CXCR4 plays a critical role in regulating EPCs homing. Here, we studied the effect of Foxc2 overexpression on CXCR4 expression and the homing capacity of EPCs as well as the EPCs-mediated therapeutic benefit after artery injury.**

Methods: **Bone marrow-derived EPCs were transfected with Foxc2 expression vector (Foxc2-EPCs) or empty control vector (Ctrl-EPCs) and examined 48 hours later. CXCR4 expression of EPCs was detected by flow cytometry and quantitative reverse transcriptase-polymerase chain reaction. The migration of EPCs toward SDF-1α was evaluated in a transwell migration assay, and the adhesion to fibronectin was determined using a static adhesion assay. For in vivo studies, EPCs were injected intravenously into the mice subjected to carotid injury. At 3 days after green fluorescent protein (GFP)/EPCs delivery, the recruited cells to the injury sites were detected by fluorescent microscopy. Re-endothelialization and neointimal formation were, respectively, assessed by Evans blue dye at 7 days and by the morphometric analysis for neointima and media area ratio (N/M) at 28 days after EPCs transfusion.**

Results: **Foxc2 overexpression significantly increased the surface expression of CXCR4 on EPCs (about 1.9-fold of Ctrl-EPCs, $P < .05$). Foxc2-EPCs showed an increased migration toward SDF-1α ($P < .05$); Foxc2 overexpression increased also the adhesion capacity of EPCs ($P < .05$). In vivo, the number of recruited GFP cells was significantly higher in the mice transfused with Foxc2-GFP/EPCs compared with Ctrl-GFP/EPCs (about 2-fold of Ctrl-GFP/EPCs). The degree of re-endothelialization was higher in mice transfused with Foxc2-EPCs compared with Ctrl-EPCs (90.3% ± 1.6% vs 57.2% ± 1.3%; $P < .05$). Foxc2-EPCs delivery resulted in a greater inhibition of neointimal hyperplasia than Ctrl-EPCs administration (N/M: 0.38 ± 0.03 vs 0.67 ± 0.05, $P < .05$). Preincubation with CXCR4-Ab, AMD3100, or LY294002 significantly attenuated the enhanced in vitro and in vivo effects of Foxc2-EPCs.**

Conclusions: **Our findings indicate that Foxc2 overexpression increases CXCR4 expression of EPCs and efficiently enhances the homing potential of EPCs, thereby improving EPCs-mediated therapeutic benefit after endothelial injury. Foxc2 may be a novel molecular target for improving the therapeutic efficacy of EPCs transplantation. (J Vasc Surg 2011;53:1668-78.)**

Clinical Relevance: **Although endothelial progenitor cells (EPCs) are capable of reducing the postintervention complications and increasing ischemic neovascularization, the extent of EPCs homing was shown to be rather low in most clinical settings, greatly limiting the effectiveness of EPCs therapy. In the present study, we report that Foxc2 overexpression enhances the homing capacity of EPCs and thereby improves the EPCs-mediated therapeutic benefit after artery injury. Our results suggest that Foxc2 may hopefully become a novel therapeutic molecular target for improving therapeutic effectiveness of EPCs delivery. On the other hand, some risk factors for coronary artery disease impair CXCR4 signaling. For instance, EPCs from diabetes mellitus or coronary artery disease patients exhibited reduced CXCR4 expression and diminished the therapeutic potential of autologous transplantation. Up-regulation of CXCR4 signaling by Foxc2 overexpression might compensate for the reduced effects, and thereby improve the therapeutic efficacy of autologous EPCs transplantation.**

Endothelial disruption or dysfunction is vital to the initiation and progression of numerous vascular diseases. Thus, maintenance of endothelial integrity and promotion of early re-endothelialization are of paramount importance for reducing cardiovascular diseases and the postintervention complications. Accumulating evidence suggests that endothelial progenitor cells (EPCs) mobilized or transfused are capable of facilitating endothelial repair through direct differentiation into endothelial cells[1,2] and/or via the paracrine mechanisms.[3,4] The vasoregenerative effects of EPCs depend on their homing to the vascular injury sites. Indeed, the number of recruited EPCs appears to be related to not only their circulating numbers but the func-

From the Institute of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Supported by a grant from National Natural Science Foundation of China (No. 30570725).

Competition of interest: none.

Reprint requests: Qiurong Ruan, Institute of Pathology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Da Dao, Wuhan 430030, Hubei Province, P. R. China (e-mail: ruanqiurong@sina.com).

The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00

doi:10.1016/j.jvs.2011.01.044

tional properties of EPCs homing. Studies show that CXCR4 is a key molecule in regulating EPCs homing and recruitment.[5-8] Emerging evidence exists for the impaired CXCR4-dependent homing and the low engraftment of EPCs due to some risk factors for coronary artery disease.[9,10] These underline the need for new strategies capable of increasing the CXCR4-mediated homing potential of EPCs.

The Foxc2 protein, a member of the Forkhead/Fox transcription factor family, is essential for the cardiovascular system. Foxc2 plays an important role in the combinatorial regulation of endothelial gene expression during embryonic development.[11] Foxc2 is recognized as a novel regulator of angiogenesis via induction of integrin β3[12] and angiopoietin-2 expression.[13] Foxc2 might be involved in the angiogenesis under pathologic conditions, just as recent studies revealed that Foxc2 expression is associated with the human heart failure[14] and increased in periinfarcted zones of the rat left ventricle.[15] Notably, Foxc2 directly induces the expression of CXCR4 in endothelial cells.[16] Recently, it is accepted that the phenotypic and functional behavior of endothelial colony forming cells (ECFCs, also called "late" EPCs) is very similar to mature endothelial cells.[17] Until now, there are no data showing the effect of Foxc2 on the functional properties of EPCs. Based on the effect of Foxc2 on the properties of endothelial cells, we sought to determine whether Foxc2 may affect CXCR4 expression and the homing capacity of EPCs as well as the EPCs-mediated endothelial repair.

METHODS

Animals. Male wild type C57BL/6J mice (25-35g) were obtained from the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Green fluorescent protein (GFP) transgenic C57BL/6J mice (25-35g) were from Jackson Laboratory (Bar Harbor, Me). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Animal Use Subcommittee at the Huazhong University of Science and Technology, P. R. China.

Preparation of bone marrow-derived EPCs. Bone marrow-derived EPCs were isolated and cultured as previously described in detail.[18] Briefly, bone marrow was aspirated from femurs and tibias of mice. Isolated mononuclear cells were cultured on a fibronectin-coated dish in endothelial cell basal medium-2 (EBM-2) supplemented with endothelial growth medium-SingleQuots (Clonetics, San Diego, Calif). After 24 hours, nonadherent cells were removed. Adherent cells were cultured for further 15 to 21 days with medium refreshment every second day, which gave EPCs.

Flow cytometry analysis. Adherent cells were trypsinized and incubated with phycocerythrin (PE)-conjugated anti-CD34, PE-conjugated anti-CD45 (Biolegend, San Diego, Calif), and PE-conjugated anti-CXCR4 (BD Biosciences Pharmingen, San Diego, Calif) for 45 min at 4°C. Flow cytometry was performed using FACS-Calibur cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Immunofluorescence analysis. Cells were fixed with 2% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, and blocked with 0.5% bovine serum albumin (BSA). Slides were incubated with rabbit anti-vWF, CD31, VE-cadherin, and CXCR4 Abs (all from Santa Cruz Biotechnology, Santa Cruz, Calif), then with Rhodamine-labeled goat anti-rabbit secondary Abs (Molecular Probes; Invitrogen, Carlsbad, Calif). The slides were counterstained for 5 minutes with Hoechst 33342 (Sigma-Aldrich, St. Louis, Mo). A fluorescence microscopy was used to analyze the antibody binding.

Uptake of DiI-acLDL. Cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (DiI-acLDL; 0.02 mg/mL; Invitrogen) at 37°C for 2 hours. After fixed, the cells were incubated with Hoechst 33342. Dual positive cells were observed with a fluorescent microscope.

Matrigel tube formation assay. Ninety-six well plates were coated with 40 μL of matrigel (BD Biosciences, Franklin Lakes, NJ). After 45 minutes of polymerization at 37°C, cells were plated at 1×10^4 cells/well and incubated for 24 hours at 37°C. Tube-like structures were observed under an inverted phase-contrast microscope.

Plasmid transfection. Mouse Foxc2 expression vector was as described,[19] kindly provided by Dr Omoteyama K of Nihon University School of Dentistry. EPCs were transiently transfected by using FuGENE HD transfection reagent (Roche, Penzberg, Germany) with pooled Foxc2 expression vector (Foxc2-EPCs) or with empty control vector (Ctrl-EPCs), following the reagent manufacturer's protocol. Briefly, the plasmid DNA solution was mixed with the transfection reagent in a DNA: transfection reagent ratio of 2:5 (μg/μL) for 15 minutes and then added to the culture medium. Forty-eight hours after transfection, the cells were used in the different assays.

Adhesion assays. EPCs were stimulated with or without 100 ng/mL SDF-1α (Peprotech, Rocky Hill, NJ) for 10 minutes before the assays. 1×10^5 EPCs were seeded on each well of a 24-well plate, precoated with fibronectin. After 30 minutes of incubation at 37°C, plates were vigorously washed to remove nonadherent cells. After being fixed with 2% PFA, adherent cells were stained with Hoechst 33342. The number of adherent cells was quantified with a fluorescent microscope in five randomly selected microscope fields (×200) per well.

Migration assay. EPCs (2×10^4 in 100 μL) were added to the upper compartment of the transwell system (Corning, Corning, NY). Six hundred microliters of serum-free basal medium containing phosphate-buffered saline (PBS), or 100 ng/mL SDF-1α was added to the lower compartment. After 18 hours at 37°C, the filter was fixed with 2% PFA and the upper side carefully cleaned with a cotton swab. For quantification, the cells on the lower side were stained with 0.1% crystal violet and counted in six randomly chosen fields (×200) under a microscope.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was extracted with TRIzol reagent (Invitrogen) and reversely transcribed using

ReverTra Ace Kit (Toyobo, Osaka, Japan), and qRT-PCR was performed on an Mx3000P real-time PCR system using SYBR Green Real-time PCR Master Mix (Toyobo). Primer pairs for Foxc2,[19] CXCR4,[20] and glyceraldehyde-3-phosphate dehydrogenase[19] were used.

Western blot analysis. EPCs protein was harvested by cell lysis buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The following antibodies were used: rabbit anti-CXCR4, CD31, VE-cad, FLK-1, β-actin, phospho-Akt, and Akt Abs (all from Santa Cruz Biotechnology), and rabbit anti-Foxc2 Ab (Abcam, Cambridge, United Kingdom). Proteins were visualized with horseradish peroxidise-conjugated anti-rabbit IgG (Cell Signaling Technology, Boston, Mass), followed by use of the enhanced chemiluminescent reagents (Thermo, Fisher, Rockford, Ill). To detect SDF-1α stimulated phosphorylation of Akt, EPCs were preincubated with 100 ng/mL SDF-1α for 10 minutes before harvesting protein.

Mouse carotid injury and EPCs transfusion. For all surgical procedures, mice were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital (Sigma). Carotid injury was established as described previously.[21] Briefly, a 0.014-inch-percutaneous transluminal coronary angioplasty flexible guidewire was introduced into the left common carotid via the external carotid artery and withdrawn/reinserted three times. The mice received 100 μL of PBS, 5×10^5 Ctrl-EPCs, or 5×10^5 Foxc2-EPCs by tail vein injection within 10 minutes following induction of vascular injury. In some experiments, EPCs were incubated with CXCR4 monoclonal blocking Ab (10 μg/mL; BD Biosciences Pharmingen), isotype control (Chemicon/Millipore, Billerica, Mass), or AMD3100 (10 μM; Sigma Aldrich), or LY294002 (10 μM; Cell Signaling Technology, Danvers, Mass) for 30 minutes at 37°C prior to the injection.

Histologic assessment. For assessment of re-endothelialization, animals were perfused with Evans blue dye (Sigma) at 7 days after EPCs delivery, as described previously.[21] Planimetric analysis was performed to calculate the ratio of re-endothelialized area, defined as an area not stained with Evans blue for the entire luminal surface area of the injured artery. For morphometric analysis of neointima formation, at 28 days following surgery, the injured carotid arteries were fixed, embedded in paraffin, serially cut into 5-μm sections, and every 20th section stained with hematoxylin and eosin. The neointima and media cross-sectional area ratio (N/M) was measured on 30 to 35 sections per animal using the image analysis software. To examine the homing capacity, at 3 days after delivery of EPCs from GFP/mice, a subset of perfusion-fixed carotid arteries were opened longitudinally and directly assessed for the number of GFP cells by en face fluorescence microscopy. The cryosections of the other carotid arteries were prepared and observed for the number of adherent GFP cells by the cross-section fluorescence microscopy.

Statistical analysis. All data are expressed as mean ± SEM. For analysis of differences between two groups, a Student's *t* test was performed. Differences between multiple groups were analyzed by one-way analysis of variance with a post hoc least significant difference analysis. A value of $P < .05$ was considered statistically significant. Statistical analysis was conducted using SPSS 13.0 software (SPSS, Chicago, Ill).

RESULTS

Characterization of endothelial progenitor cells. Bone marrow derived–mononuclear cells were isolated and cultured for 15 to 21 days, which exhibited a cobblestone-like morphology (Fig 1, *A* and *B*). Immunofluorescence demonstrated that the majority of adherent cells expressed the endothelial cell markers, including CD31 (Fig 1, *C*), VE-cad (Fig 1, *D*), and vWF (Fig 1, *E*). Flow cytometric analysis revealed that only very few cells expressed the hematopoietic marker CD34 (Fig 1, *G*) or leukocytic marker CD45 (Fig 1, *H*). Western blot analysis further confirmed the expression of endothelial cell markers, including CD31, VE-cad, and FLK-1 (Fig 1, *I*). Moreover, the majority of adherent cells (>95%) were found positive for uptake of DiI-acLDL (Fig 1, *J*). In addition, these cells were capable of assembling into tube-like structures when plated in matrigel (Fig 1, *K*). On the basis of these morphologic and functional characteristics, the cells in our study were confirmed EPCs characterized as ECFCs.[17,22]

Confirmation of Foxc2 overexpression in EPCs. At 48 hours posttransfection, the transcription and expression of Foxc2 in EPCs were confirmed by qRT-PCR and Western blot. Neither the transfection reagent nor the empty control vector affected Foxc2 expression in EPCs, whereas the transfection with Foxc2 expression vector efficiently induced the upregulation of Foxc2 mRNA (about 4.5-fold of control value [$P < .05$]; Fig 2, *A*). Furthermore, Western blot showed that Foxc2 protein levels in Foxc2-EPCs were increased to 3.5 folds of the other 3 groups ($P < .05$; Fig 2, *B*).

Foxc2 overexpression upregulates CXCR4 expression of EPCs. To explore whether Foxc2 overexpression may affect CXCR4 expression of EPCs, we first evaluated the surface expression of CXCR4 on EPCs using flow cytometry. As shown in Fig 2, *C*, Foxc2 overexpression significantly increased surface expression of CXCR4 on EPCs ($P < .05$). Fluorescence microscopy and Western blot analysis further confirmed that Foxc2 overexpression upregulated CXCR4 expression of EPCs (Fig 2, *D* and *E*). qRT-PCR analysis showed CXCR4 mRNA expression in Foxc2-EPCs was about two-fold of Ctrl-EPCs or EPCs (Fig 2, *F*), suggesting that upregulation of CXCR4 expression appeared to be related to an increase in CXCR4 mRNA levels.

Foxc2 overexpression increases the SDF-1α–induced migration and adhesion of EPCs in vitro. To determine whether the induction of CXCR4 expression by Foxc2 overexpression is functionally involved in the migration and adhesion capacities of EPCs, we investigated their migration toward SDF-1α in a transwell migration assay, and their adhesion to fibronectin using an adhesion assay under static conditions. The number of spontaneous migration was not

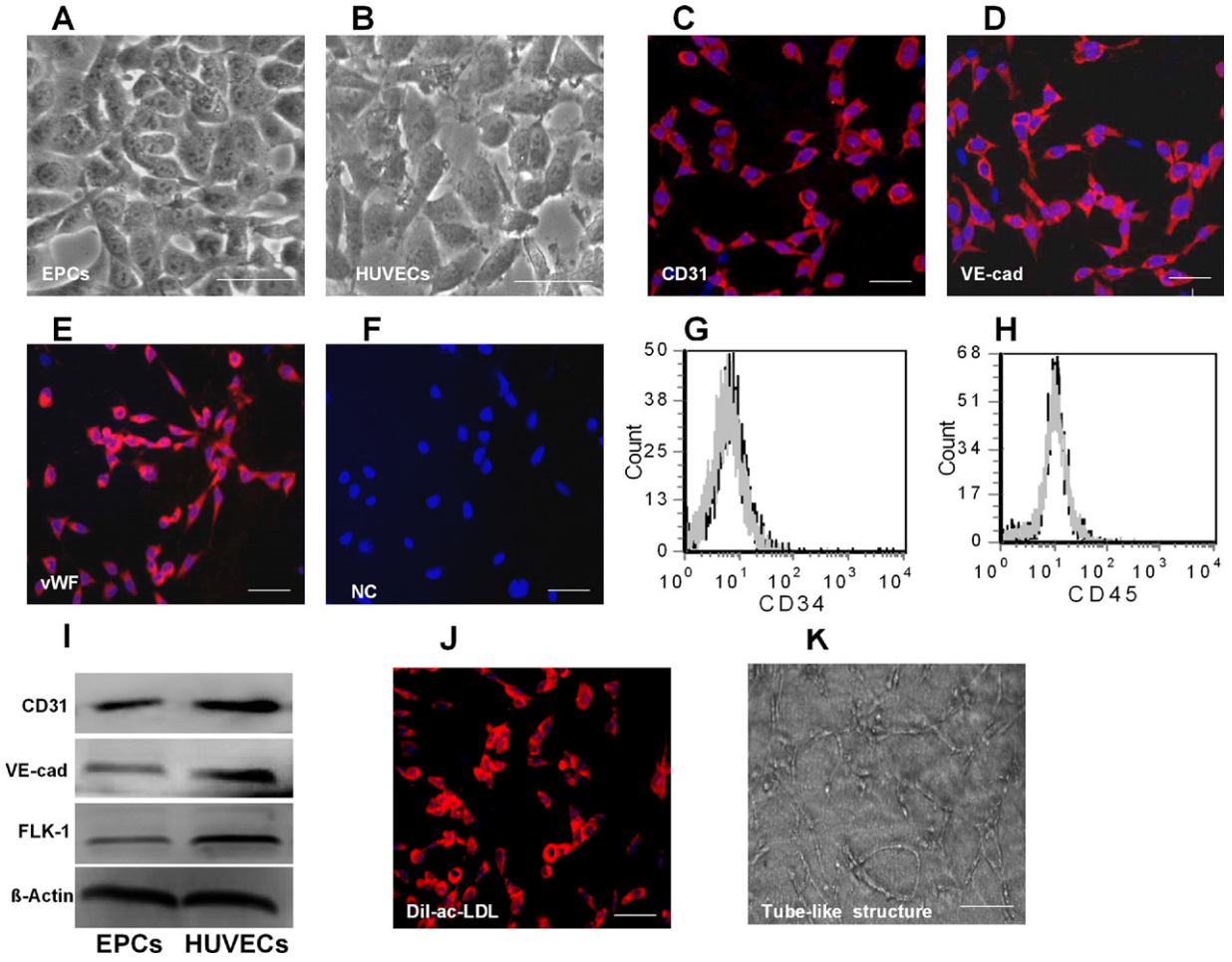

Fig 1. Morphologic and functional analysis of bone marrow-derived endothelial progenitor cells (*EPCs*). Bone marrow-derived mononuclear cells were cultured for 15 to 21 days. **A,** The confluent endothelial-like monolayers with cobblestone pattern were found after 15 days. **B,** The similar morphology was observed in human umbilical cord vein endothelial cells (*HUVECs*). **C-F,** Immunofluorescence demonstrated that the majority of the cells expressed the endothelial markers of CD31 **(C)**, VE-cadherin **(D)**, vWF **(E)** compared to isotype-NC **(F)**. Nuclei were counterstained with Hoechst 33342. **G-H,** Flow cytometry revealed that the hematopoietic (CD34; **G**) or leukocytic marker (CD45; **H**) was expressed on the minority of the cells. *Black histograms* represent specific fluorescence, and *silver histograms* the respective isotype controls. **I,** Western-blot analysis confirmed the expression of endothelial cell markers; HUVECs were used as positive controls; β-actin protein expression was used as internal standard. Culture cells were evaluated by DiI-ac-LDL incorporation **(J)** and by the formation of vascular tube-like structures on matrigel **(K)**. *Scale bars*, 100 μm **A-F** and **J**; 200 μm **(K)**.

significantly different among EPCs, Ctrl-EPCs, and Foxc2-EPCs (Fig 3, *A*). Foxc2-EPCs demonstrated an increased response to SDF-1α–mediated chemotaxis (about 1.4-fold of Ctrl-EPCs or EPCs; $P < .05$; Fig 3, *A*). The basal adhesion capacity had no obvious difference among the three groups (Fig 3, *B*). After stimulation with SDF-1α, although the adhesion capacity of EPCs or Ctrl-EPCs was increased, Foxc2-EPCs had a further enhance ($P < .05$; Fig 3, *B*).

It has been documented that after exposure to SDF-1α at high concentrations (1μg/mL and above) induces the desensitization of the cells to further SDF-1α stimulation, via the endocytosis of the cell surface CXCR4 molecule.[23] We therefore examined the effects of high SDF-1α concentrations on Foxc2-EPCs. To this end, Foxc2-EPCs were incubated overnight with 1μg/mL SDF-1α, and evaluated for the CXCR4 cell surface expression. Unexpectedly, there was only a 43% decrease in cell surface receptor expression in Foxc2-EPCs, whereas in Ctrl-EPCs this decrease reached up to 87% ($P < .05$; Fig 3, *C*). Desensitized cells were also assayed for the SDF-1α (100 ng/mL)-directed chemotaxis and adhesion. Ctrl-EPCs decreased by nearly 50% in the SDF-1α-mediated migration and adhesion, whereas these responses of Foxc2-EPCs were hardly affected (Fig 3, *D* and *E*), indicating that Foxc2-EPCs were less responsive to SDF-1α–induced desensitization.

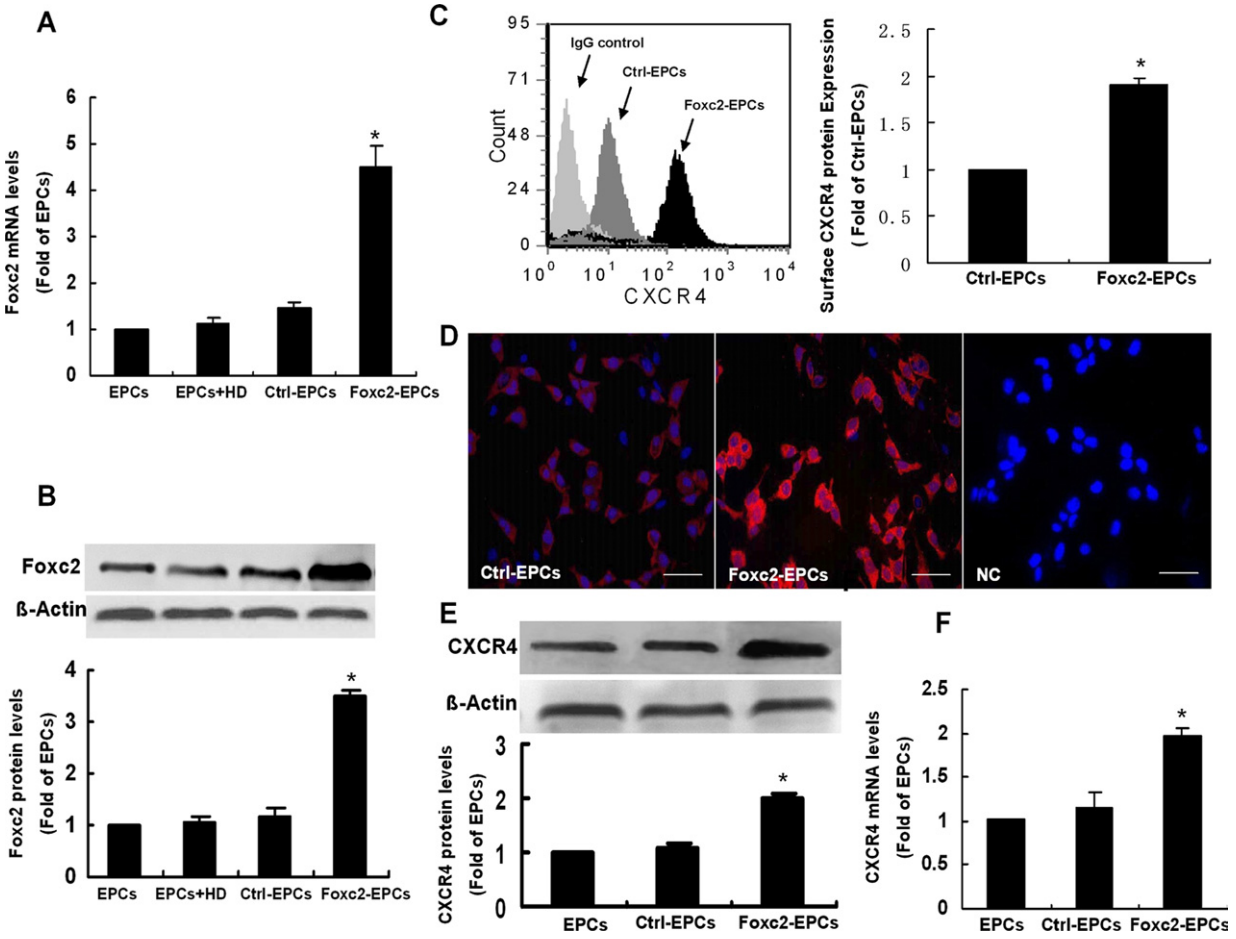

Fig 2. A-B, Liposome-mediated transfection of Foxc2 expression vector upregulated Foxc2 expression of endothelial progenitor cells (*EPCs*). At 48 hours posttransfection, the expression of Foxc2 mRNA was determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) **(A)** (n = 5 per group), and the protein expression was measured by Western blot (**B**; n = 5 per group). GAPDH mRNA and β-actin protein expression were used as internal standard. Results for EPCs transfected with HD alone (*EPCs + HD*), the empty control vector (*Ctrl-EPCs*), or Foxc2 expression vector (*Foxc2-EPCs*) were normalized to the untransfected EPCs (*EPCs*). **P* < .05 vs EPCs, EPCs + HD, or Ctrl-EPCs. **C-F,** Foxc2 overexpression induced CXCR4 expression of EPCs. **C,** Flow cytometry analysis of CXCR4 surface expression. Representative histograms for isotype IgG control EPCs (*silver*), Ctrl-EPCs (*gray*), and Foxc2-EPCs (*black*) were shown (n = 5 independent experiments). **D,** Immunofluorescence confirmed the upregulation of CXCR4 expression in Foxc2-EPCs (*middle*) compared with Ctrl-EPCs (*left*). The isotype NC was also shown. *Scale bars*, 100 μm. Western-blot **E,** and qRT-PCR **(F)** showing CXCR4 expression of EPCs, Ctrl-EPCs, and Foxc2-EPCs (n = 5 per group). GAPDH mRNA and β-actin protein expression were used as internal standard. **P* < .05 vs Ctrl-EPCs or EPCs.

Upregulation of CXCR4/PI3K/Akt signaling was involved in the effects of Foxc2 overexpression on EPCs migration and adhesion. Based on the evidence that PI3K/Akt is a known downstream target of CXCR4 receptor,[24] we determined whether Foxc2 overexpression affects the activity of PI3K/Akt signaling. Western blot analysis revealed that the basal Akt-phosphorylation was not significantly different between Ctrl-EPCs and Foxc2-EPCs, whereas SDF-1α–stimulated increase of Akt-phosphorylation in Foxc2-EPCs exceeded that in Ctrl-EPCs (Fig 3, *F*). Furthermore, the increased Akt-phosphorylation in Foxc2-EPCs was inhibited by preincubation with CXCR4-Ab, CXCR4 antagonist AMD3100, or PI3K inhibitors LY294002 (Fig 3, *F*). Next, we tested the ability of CXCR4-Ab, AMD3100, or LY294002 to impair the migration and adhesion function of Foxc2-EPCs. As observed in the above results, Foxc2 overexpression increased the migration and adhesion capacities of EPCs. However, the increased effects were reduced by pretreatment of Foxc2-EPCs with the CXCR4-Ab, AMD3100, or LY294002 (Fig 3, *G* and *H*).

Foxc2 overexpression contributes to EPCs homing and recruitment into the sites of vascular injury. To test the hypothesis that the enhanced in vitro capacities of EPCs

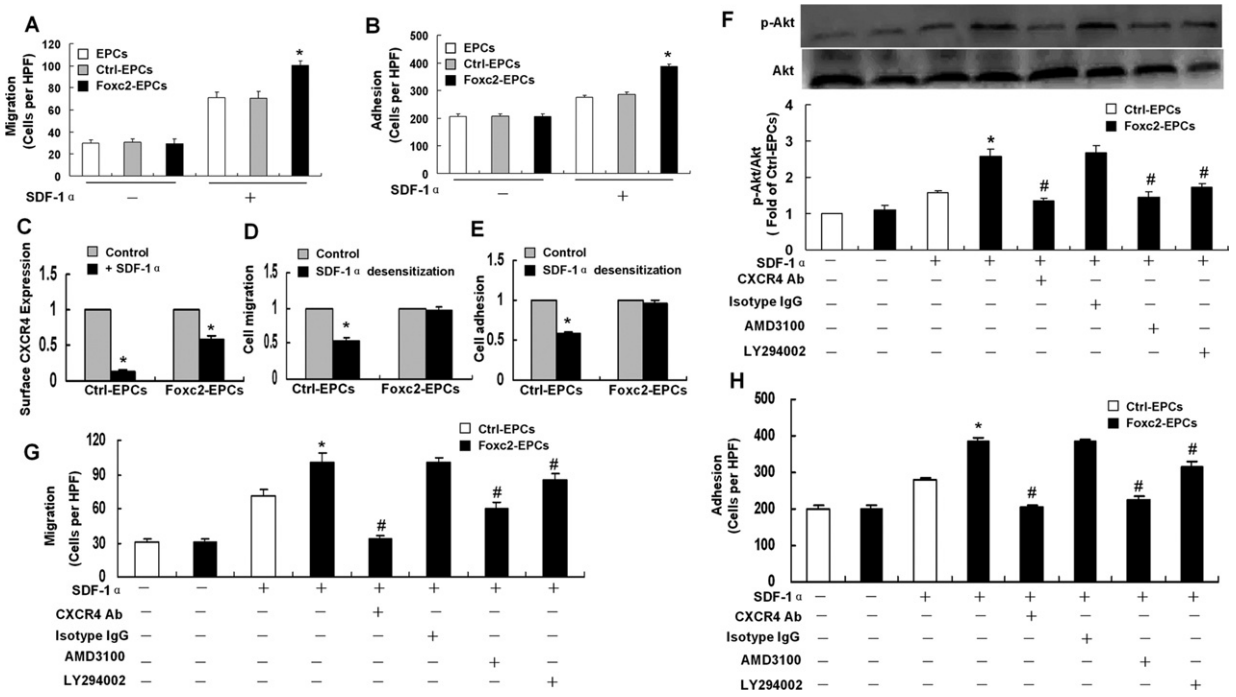

Fig 3. A-B, Effects of Foxc2 overexpression on SDF-1α-induced migration and adhesion of endothelial progenitor cells (*EPCs*) in vitro. **A,** Migration assay: At 48 hours after transfection, the cells were allowed to migrate to the lower compartment of transwell migration system, containing 100 ng/mL SDF-1α for 18 hours. The migrated cells were stained and counted under a microscope. n = 5 independent experiments. **P* < .05 vs EPC + SDF-1α or Ctrl-EPIC + SDF-1α. **B,** Adhesion assay: The cells pretreated with or without SDF-1α stimulation were allowed to adhere to fibronectin for 30 minutes. Adherent cells were stained and observed under a fluorescent microscope. n = 5 independent experiments. **P* < .05 vs EPC + SDF-1α or Ctrl-EPIC + SDF-1α. **C-E,** Response of Foxc2-EPCs to SDF-1α–induced desensitization. Foxc2-EPCs and Ctrl-EPCs were incubated overnight with SDF-1α (1μg/mL), and evaluated for the surface CXCR4 expression by flow cytometry **(C)** and the SDF-1α (100ng/mL)-induced migration **(D)** and adhesion **(E)**. Results for the desensitized cells were normalized to the untreated cells. n = 5 independent experiments. **P* < .05 compared with the untreated control cells. **F-H,** Upregulation of CXCR4/PI3K/Akt signaling was involved in the enhanced in vitro migration and adhesion function of EPCs by Foxc2 overexpression. **F,** Representative Akt phosphorylation of EPCs with or without stimulation by SDF-1α was measured by Western blot (n = 5 per group); quantification was expressed as p-Akt/Akt ratio. Data were normalized to Ctrl-EPCs without SDF-1α stimulation. Quantification analysis for SDF-1α induced migration **(G)** and adhesion **(H)** of EPCs after treatment with AMD3100, CXCR4-Ab, or LY294002 (n = 5 per group). **P* < .05 vs Ctrl-EPCs + SDF-1α; #*P* < .05 vs Foxc2-EPCs + SDF-1α.

achieved by Foxc2 overexpression would translate into the improved in vivo homing function, the mice subjected to carotid injury were intravenously injected with PBS, Foxc2-GFP/EPCs, or Ctrl-GFP/EPCs. Three days later, cross-section or en face fluorescent microscopy showed the delivered GFP cells were recruited at injury sites. The number of recruited GFP cells was significantly higher in the mice transfused with Foxc2-GFP/EPCs compared with Ctrl-GFP/EPCs (about two-fold of Ctrl-GFP/EPCs; Fig 4, *A* and *B*). Homing of transfused cells was strictly restricted to the injury sites, with no detectable cells in uninjured parts or in the contralateral vessel. Importantly, the Foxc2-overexpressing-induced effect was significantly reduced by pretreatment of Foxc2-EPCs with CXCR4-Ab, AMD3100, or LY294002 for 30 minutes prior to their injection (Fig 4, *A* and *B*).

Foxc2 overexpression enhances the therapeutic benefit of EPCs after artery injury. To assess the effect of Foxc2-EPCs delivery on endothelial repair, re-endothelialization was examined using Evens blue staining of the injury vessels. At 7 days after EPCs delivery, the degree of re-endothelialization was significantly higher in mice transfused with Ctrl-EPCs compared with PBS ($P < .05$; Fig 5, *A* and *B*). Nevertheless, Foxc2-EPCs delivery further increased the degree of re-endothelialization relative to Ctrl-EPCs (90.3% ± 1.6% vs 57.2% ± 1.3%, $P < .05$; Fig 5, *A* and *B*). Further experiments indicated that more GFP cells were incorporated into the CD31 positive endothelial layer at 14 days after Foxc2-GFP/EPCs compared with Ctrl-EPCs delivery (46.67% ± 7.09% vs 31.50% ± 5.26%, $P < .05$; Fig 5, *C*). Next, to evaluate whether Foxc2 overexpression enhanced the ability of EPCs to inhibit neointimal

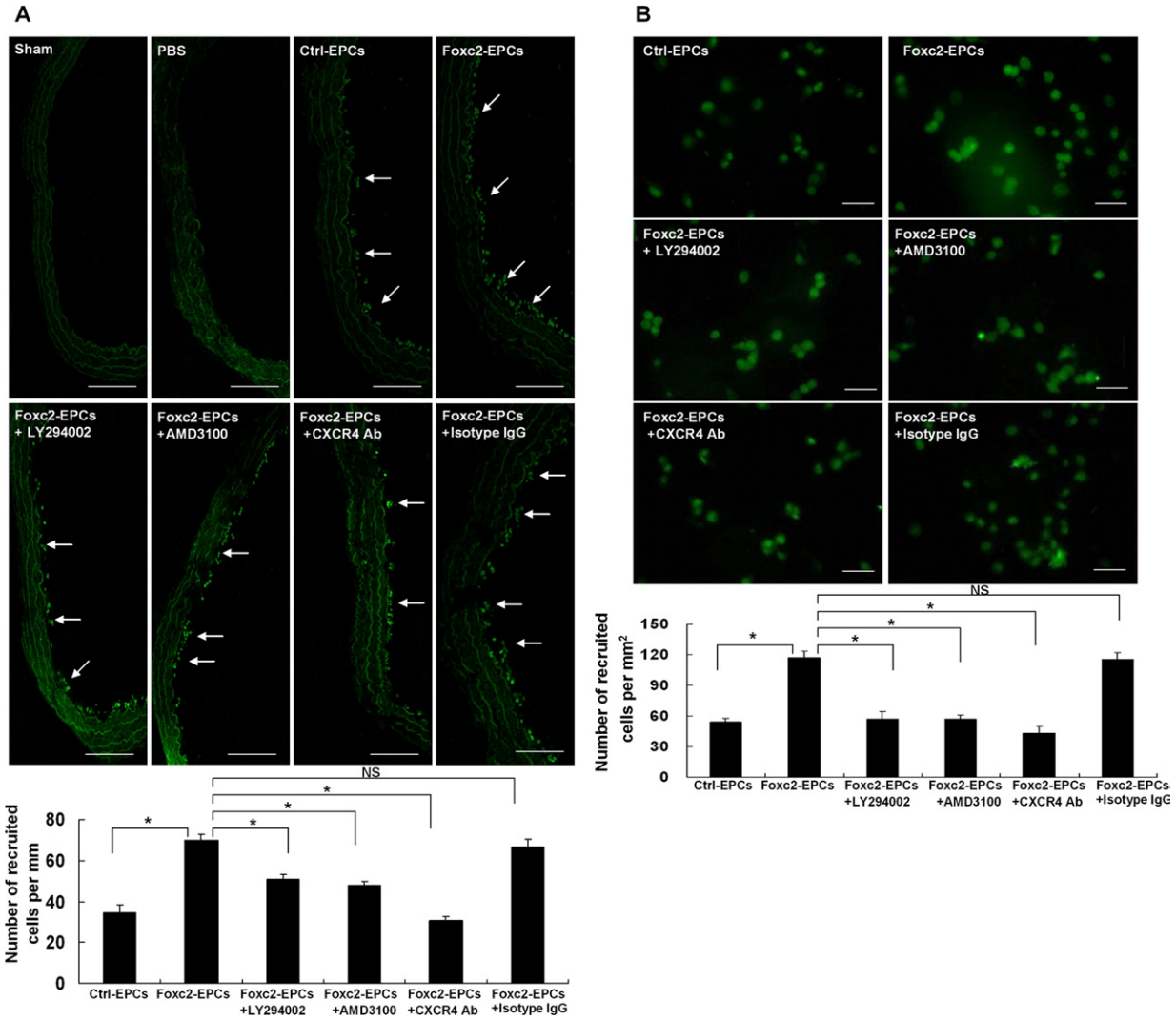

Fig 4. Effect of Foxc2 overexpression on homing and recruitment of endothelial progenitor cells (*EPCs*) into the sites of vascular injury and its correlation with CXCR4/PI3K/Akt signaling. The mice subjected to carotid injury were transfused with phosphate-buffered saline (PBS), Ctrl-green fluorescent protein (GFP)/EPCs, or Foxc2-GFP/EPCs with or without pretreatment with AMD3100, CXCR4-Ab, or LY294002. Three days later, the number of recruited cells to the injury sites was detected by cross-section or en face fluorescent microscopy. **A,** Representative photomicrographs of the cross-section fluorescent microscopy for the injured carotid arteries and the contralateral uninjured carotid arteries (sham). *Scale bars*, 200 μm. And the analysis of the number of recruited cells per mm (luminal circumference). n = 3 to 5 per group. *$P < .05$. **B,** Representative photomicrographs of the injured carotid arteries under an en face fluorescent microscopy. *Scale bars*, 100 μm. And the analysis of the recruited cell number per mm^2. n = 5 per group. *$P < .05$.

hyperplasia, the carotid arteries were harvested 28 days after EPCs delivery. Wire-induced injury resulted in the prominent neointimal formation in the arteries from PBS-injected mice (N/M: 1.94 ± 0.06; Fig 6). Ctrl-EPCs delivery led to a 65% reduction in N/M compared with PBS control ($P < .05$). However, the inhibitory effect of Foxc2-EPCs delivery was greater than Ctrl-EPCs (0.38 ± 0.03 vs 0.67 ± 0.05, $P < .05$; Fig 6). Finally, preincubation with CXCR4-Ab, AMD3100, or LY294002 significantly attenuated the enhanced therapeutic potential of Foxc2-EPCs for promoting re-endothelialization and inhibiting neointimal formation (Figs 5, *A* and *B*, and 6).

DISCUSSION

It is accepted that EPCs are capable of enhancing re-endothelialization and diminishing neointimal formation. In the context of the regenerative arterial remodeling, an adequate homing of EPCs plays an important role. However, the extent of homing was shown to be rather low in most experimental and clinical studies, which is a hurdle

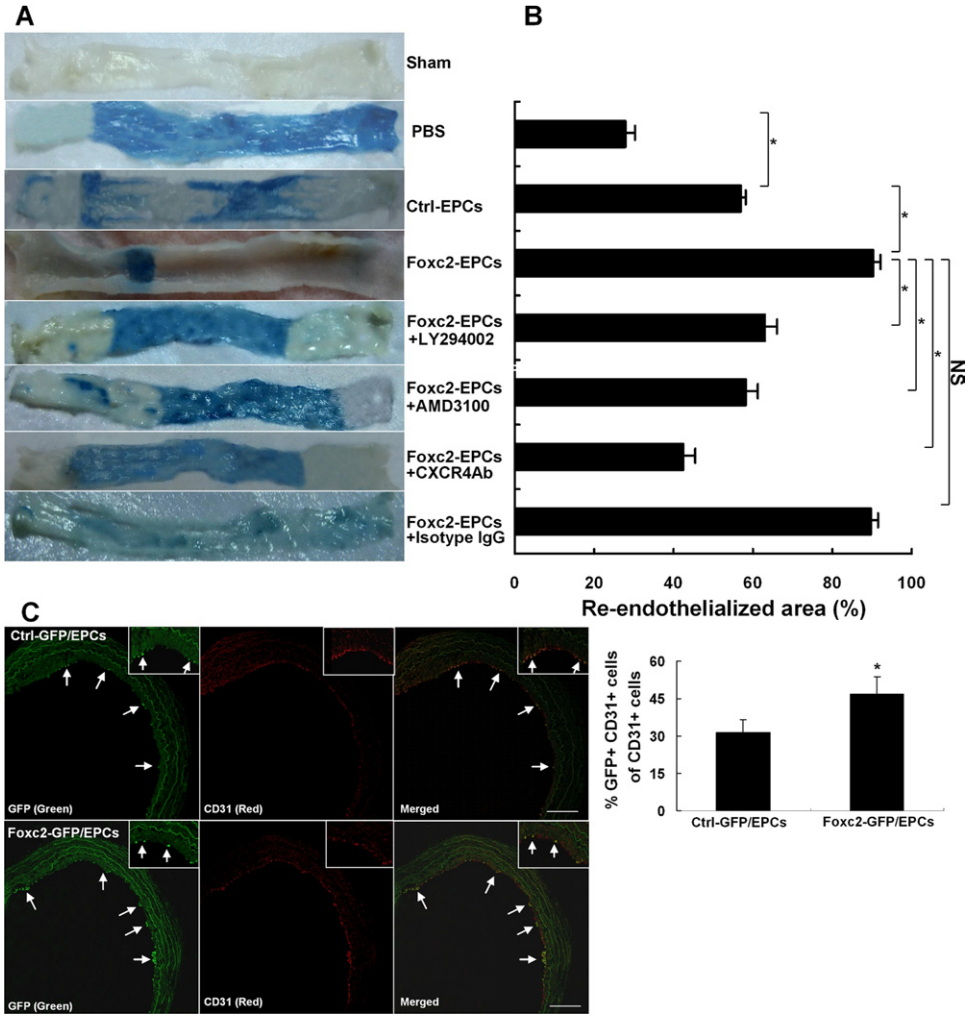

Fig 5. Foxc2 overexpression facilitated endothelial progenitor cells (*EPCs*)-mediated re-endothelialization after artery injury. **A,** Representative photographs of injured carotid arteries stained by Evans blue. **B,** Quantification of the re-endothelialized area assessed by the percentage of Evans blue nonstaining area for the entire injured area. n = 5 per group. **P* < .05. **C,** Incorporation of EPCs into the regenerated endothelium. At 14 days after Ctrl-green fluorescent protein (*GFP*)/EPCs or Foxc2-GFP/EPCs delivery, GFP cells (*green*) were detected within the endothelial layer identified by immunostaining for CD31 (Rhodamine, *red*). *Scale bar*, 200 μm. And the analysis of the percent of GFP positive endothelial cells in the cross-section of injured arteries. n = 3 to 4 per group. **P* < .05.

that has still not been adequately addressed or overcome so far. In the present study, Foxc2 overexpression efficiently increased EPCs homing to the sites of vascular injury, thereby resulting in enhanced re-endothelialization and attenuated neointimal formation. In vitro, Foxc2 overexpression markedly upregulated CXCR4 expression in EPCs and increased the migration and adhesion capacities of EPCs. Moreover, the enhanced in vitro and in vivo function by Foxc2 overexpression was associated with the upregulation of CXCR4 and the activation of PI3K/Akt signal pathway. These findings provide novel insights into the effects of Foxc2 on vascular homeostasis.

We demonstrated that Foxc2 overexpression markedly increased the expression of CXCR4 in EPCs. Consistent with our finding, hypoxia,[25] ex vivo short-time culture,[26] or cytokine expansion culture[27,28] may upregulate CXCR4 expression of the stem cells. However, the upregulation of CXCR4 is due to translocation of intracellular CXCR4 to the cell surface. Here, we particularly observed that Foxc2 overexpression stimulated CXCR4 gene transcription, suggesting that the upregulation of CXCR4 expression by Foxc2 overexpression was at least partially due to the de novo synthesis. Integrative genomic analyses of CXCR4 gene indicate that FOX-binding sites of mammalian CXCR4 orthologs is almost completely conserved.[29] Furthermore, Foxc2 directly regulates CXCR4 expression in endothelial cells by activating its promoter.[16] Therefore, these findings support the transcriptional upregulation of

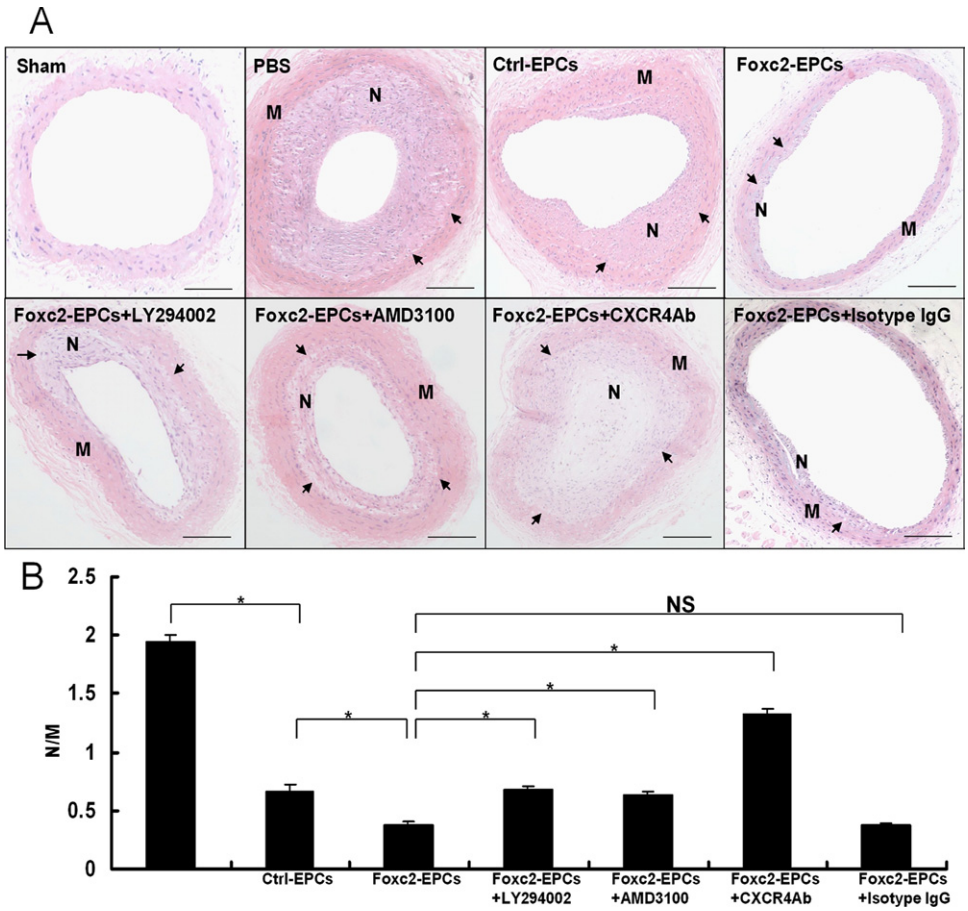

Fig 6. Foxc2 overexpression enhanced the capacity of endothelial progenitor cells (*EPCs*) to inhibit neointimal formation. The cross-sections of carotid arteries at 28 days postinjury were stained with hematoxylin and eosin. Media (*M*) and neointima (*N*) are labeled for reference. *Arrows* indicate the internal elastic lamina. **A,** Representative cross-sections of the carotid arteries from the mice transfused with phosphate-buffered saline (PBS), Ctrl-EPCs, or Foxc2-EPCs with or without AMD3100, CXCR4-mAb, or LY294002. *Scale bars*, 200 μm. **B,** Morphometric analysis of the cross-section N/M, n = 5 per group, $*P < .05$.

CXCR4 by Foxc2 overexpression in EPCs. Interestingly, there was hardly any response to SDF-1α–induced desensitization in Foxc2-EPCs. It is well documented that the cell desensitization results from the internalization of CXCR4 after exposure to SDF-1α.[23] Our data showed that in Foxc2-EPCs, there was only a 43% decrease in cell surface CXCR4 expression compared with an 87% decrease in Ctrl-EPCs after exposure to SDF-1α. Thus, we guess that the less desensitization of Foxc2-EPCs may be explained by constant upregulation of CXCR4 compensating for internalization of CXCR4 and partially overcoming the desensitization to SDF-1α. The less desensitization might contribute to the enhanced therapeutic benefit of Foxc2-EPCs.

CXCR4 is a key molecule in regulating the therapeutic potential of EPCs. Recently, it has been reported that the differences in donor CXCR4 expression levels are correlated with the therapeutic outcome of angiogenic treatment with EPCs.[30] More important, CXCR4 expression determines the functional activity of bone marrow-derived mononuclear cells for therapeutic neovascularization in acute ischemia.[31] To explore the therapeutic potential of EPCs that express high levels of CXCR4 by Foxc2 overexpression in the cardiovascular diseases of endothelial injury, we produced a mouse model of carotid injury. Our results showed that Foxc2 overexpression significantly enhanced the therapeutic potential of EPCs for promoting re-endothelialization and inhibiting neointimal formation, and the improved therapeutic benefit was clearly impaired by CXCR4-Ab or AMD3100. Consistent with our results, Chen et al reported that the direct upregulation of CXCR4 expression in human EPCs by gene transfer results in an improvement of re-endothelialization capacity.[32]

Homing is a prerequisite for the transfused cells via the vascular route to exhibit their activity in the target tissue. It is now evident that SDF-1 expression is markedly upregulated in the injured areas early after endothelial denuda-

tion,[33,34] and the SDF-1/CXCR4 axis plays a crucial role in homing of EPCs to the sites of denudation.[35] Concerning how Foxc2 overexpression modulates the therapeutic potential of EPCs, we hypothesized that the enhanced therapeutic potential of Foxc2-EPCs might be exerted via the increased CXCR4-dependent homing. Indeed, Foxc2 overexpression enhanced the SDF-1α-induced migration and adhesion in vitro. In vivo, Foxc2-EPCs exhibited the increased homing and recruitment into the injury sites. Moreover, both the enhanced in vitro and in vivo functional properties of Foxc2-EPCs can be attenuated by CXCR4-Ab or AMD3100, suggesting the enhanced homing of EPCs by Foxc2 overexpression was CXCR4-dependent. Further, more GFP cells were observed within the CD31 positive endothelial layer 14 days after Foxc2-GFP/EPCs delivery, suggesting that the delivered Foxc2-EPCs were actually incorporated into the regenerated endothelial layer. Thus, it is likely that Foxc2-EPCs differentiate into endothelial cells, thereby resulting in the rapid re-endothelialization after artery injury.

It is well-known that PI3K/Akt is an important downstream of SDF-1/CXCR4 signal pathway and plays an essential role in regulating the migratory and homing potential of progenitor cells.[36] In order to understand the molecular mechanism, we hypothesized that PI3K/Akt signaling might be related to the enhanced EPCs function by Foxc2 overexpression mentioned above. Our data showed that Akt phosphorylation was more responsive to SDF-1α in Foxc2-EPCs compared with Ctrl-EPCs. Moreover, the enhanced in vitro function as well as the improved in vivo homing potential and therapeutic benefit of Foxc2-EPCs could be attenuated by PI3K inhibitors LY294002. These results suggest that the improved effects by Foxc2 overexpression are mainly dependent on the CXCR4-mediated PI3K/Akt signal pathway.

There are several limitations in our study. First, impairment of EPCs function by cardiovascular risk factors has to be taken into account when considering EPC-based therapy. However, we did not investigate the effect of Foxc2 overexpression on dysfunctional EPCs from the high-risk subjects. Second, it has been reported that Foxc2 is associated with the metastasis and angiogenesis of tumor.[37,38] Thus, the clinical safety of Foxc2-based therapy should still be verified. Finally, we did not investigate the influence of Foxc2 inhibition on the migration and homing of EPCs, so the definite roles of Foxc2 in the homing of EPCs remain to be clarified.

In conclusion, we demonstrated that Foxc2 overexpression enhances EPCs homing potential and therapeutic benefit of EPCs for facilitating re-endothelialization and inhibiting neointimal hyperplasia. These findings also raise the possibility that Foxc2 may potentially become a novel therapeutic molecular target for improving therapeutic benefit of EPCs transplantation. Future studies will have to examine the effect of Foxc2 overexpression on ischemic neovascularization of EPCs.

The authors are grateful to Dr Omoteyama K (Nihon University School of Dentistry) for providing the mouse Foxc2 expression vector.

AUTHOR CONTRIBUTIONS

Conception and design: RQ, LD
Analysis and interpretation: LD
Data collection: LD, YD, LW, LM, YJ, LY, QZ
Writing the article: LD
Critical revision of the article: RQ, LD, YD, LW, LM, YJ, LY, QZ
Final approval of the article: RQ, LD, YD, LW, LM, YJ, LY, QZ
Statistical analysis: LD
Obtained funding: RQ
Overall responsibility: RQ

REFERENCES

1. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 2003;93:e17-24.
2. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002;105:3017-24.
3. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003;107:1164-9.
4. O'Neill TJ 4th, Wamhoff BR, Owens GK, Skalak TC. Mobilization of bone marrow-derived cells enhances the angiogenic response to hypoxia without transdifferentiation into endothelial cells. Circ Res 2005;97:1027-35.
5. Sainz J, Sata M. CXCR4, a key modulator of vascular progenitor cells. Arterioscler Thromb Vasc Biol 2007;27:263-5.
6. Hristov M, Zernecke A, Liehn EA, Weber C. Regulation of endothelial progenitor cell homing after arterial injury. Thromb Haemost 2007;98:274-7.
7. Walter DH, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C, et al. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arterioscler Thromb Vasc Biol 2007;27:275-82.
8. Smadja DM, Bièche I, Uzan G, Bompais H, Muller L, Boisson-Vidal C, et al. PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. Arterioscler Thromb Vasc Biol 2005;25:2321-7.
9. Hamed S, Brenner B, Abassi Z, Aharon A, Daoud D, Roguin A. Hyperglycemia and oxidized-LDL exert a deleterious effect on endothelial progenitor cell migration in type 2 diabetes mellitus. Thromb Res 2010;126:166-74.
10. Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, et al. Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 2005;97:1142-51.
11. De Val S, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, et al. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. Cell 2008;135:1053-64.
12. Hayashi H, Sano H, Seo S, Kume T. The Foxc2 transcription factor regulates angiogenesis via induction of integrin beta3 expression. J Biol Chem 2008;283:23791-800.
13. Xue Y, Cao R, Nilsson D, Chen S, Westergren R, Hedlund EM, et al. FOXC2 controls Ang-2 expression and modulates angiogenesis, vascular patterning, remodeling, and functions in adipose tissue. Proc Natl Acad Sci U S A 2008;105:10167-72.

14. Hannenhalli S, Putt ME, Gilmore JM, Wang J, Parmacek MS, Epstein JA, et al. Transcriptional genomics associates FOX transcription factors with human heart failure. Circulation 2006;114:1269-76.
15. Philip-Couderc P, Tavares NI, Roatti A, Lerch R, Montessuit C, Baertschi AJ. Forkhead transcription factors coordinate expression of myocardial KATP channel subunits and energy metabolism. Circ Res 2008;102:e20-35.
16. Hayashi H, Kume T. Forkhead transcription factors regulate expression of the chemokine receptor CXCR4 in endothelial cells and CXCL12-induced cell migration. Biochem Biophys Res Commun 2008;367: 584-9.
17. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008;28:1584-95.
18. Imamura H, Ohta T, Tsunetoshi K, Doi K, Nozaki K, Takagi Y, et al. Transdifferentiation of bone marrow-derived endothelial progenitor cells into the smooth muscle cell lineage mediated by transforming growth factor-beta1. Atherosclerosis 2010;211:114-21.
19. Omoteyama K, Mikami Y, Takagi M. Foxc2 induces expression of MyoD and differentiation of the mouse myoblast cell line C2C12. Biochem Biophys Res Commun 2007;358:885-9.
20. Jujo K, Hamada H, Iwakura A, Thorne T, Sekiguchi H, Clarke T, et al. CXCR4 blockade augments bone marrow progenitor cell recruitment to the neovasculature and reduces mortality after myocardial infarction. Proc Natl Acad Sci U S A 2010;107:11008-13.
21. Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, et al. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 2003; 108:3115-21.
22. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007;109:1801-9.
23. Signoret N, Oldridge J, Pelchen-Matthews A, Klasse PJ, Tran T, Brass LF, et al. Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. J Cell Biol 1997;139: 651-64.
24. Zheng H, Dai T, Zhou B, Zhu J, Huang H, Wang M, et al. SDF-1alpha/CXCR4 decreases endothelial progenitor cells apoptosis under serum deprivation by PI3K/Akt/eNOS pathway. Atherosclerosis 2008;201:36-42.
25. Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, et al. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res 2009;104:1209-16.
26. Shiba Y, Takahashi M, Hata T, Murayama H, Morimoto H, Ise H, et al. Bone marrow CXCR4 induction by cultivation enhances therapeutic angiogenesis. Cardiovasc Res 2009;81:169-77.
27. Denning-Kendall P, Singha S, Bradley B, Hows J. Cytokine expansion culture of cord blood $CD34^+$ cells induces marked and sustained changes in adhesion receptor and CXCR4 expressions. Stem Cells 2003;21:61-70.
28. Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003;107:1322-8.
29. Katoh M, Katoh M. Integrative genomic analyses of CXCR4: transcriptional regulation of CXCR4 based on TGFbeta, Nodal, activin signaling and POU5F1, FOXA2, FOXC2, FOXH1, SOX17, and GFI1 transcription factors. Int J Oncol 2010;36:415-20.
30. Oh BJ, Kim DK, Kim BJ, Yoon KS, Park SG, Park KS, et al. Differences in donor CXCR4 expression levels are correlated with functional capacity and therapeutic outcome of angiogenic treatment with endothelial colony forming cells. Biochem Biophys Res Commun 2010;398:627-33.
31. Seeger FH, Rasper T, Koyanagi M, Fox H, Zeiher AM, Dimmeler S. CXCR4 expression determines functional activity of bone marrow-derived mononuclear cells for therapeutic neovascularization in acute ischemia. Arterioscler Thromb Vasc Biol 2009;29:1802-9.
32. Chen L, Wu F, Xia WH, Zhang YY, Xu SY, Cheng F, et al. CXCR4 gene transfer contributes to in vivo reendothelialization capacity of endothelial progenitor cells. Cardiovasc Res 2010;88:462-70.
33. Schober A, Knarren S, Lietz M, Lin EA, Weber C. Crucial role of stromal cell-derived factor-1alpha in neointima formation after vascular injury in apolipoprotein E-deficient mice. Circulation 2003;108: 2491-7.
34. Zernecke A, Schober A, Bot I, von Hundelshausen P, Liehn EA, Möpps B, et al. SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ Res 2005;96:784-91.
35. Yin Y, Zhao X, Fang Y, Yu S, Zhao J, Song M, et al. SDF-1alpha involved in mobilization and recruitment of endothelial progenitor cells after arterial injury in mice. Cardiovasc Pathol 2010;19:218-27.
36. Chavakis E, Carmona G, Urbich C, Göttig S, Henschler R, Penninger JM, et al. Phosphatidylinositol-3-kinase-gamma is integral to homing functions of progenitor cells. Circ Res 2008;102:942-9.
37. Sano H, Leboeuf JP, Novitskiy SV, Seo S, Zaja-Milatovic S, Dikov MM, et al. The Foxc2 transcription factor regulates tumor angiogenesis. Biochem Biophys Res Commun 2010;392:201-6.
38. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, et al. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci U S A 2007;104:10069-74.

Submitted Nov 7, 2010; accepted Jan 16, 2011.